CHAPTER 1:

GENERAL INTRODUCTION.

STARCH AS A RAW MATERIAL.

In Western Europe approximately two thirds of the total starch production is used in the food and beverage industries (Koch and Röper 1988). It is directly applied as thickener in sauces, custards and desserts. After enzymatic hydrolysis it is also used as sweetener in drinks and confectionery.

One third of the total starch production is used for non-food purposes, e.g. for sizing of paper and board and as an adhesive in the paper, packaging and textile industry. A small part (10 % of the total starch production) is used as a raw material in the chemical industry (Koch and Röper 1988). Polyols, (amino)acids, cyclodextrins, fructose, antibiotics and related compounds are obtained from starch via various fermentation processes (Tubb 1986, Koch and Röper 1988, Röper and Koch 1988). Further application of starch in the chemical industry may result from effective competition with cellulose and crude oil, two other raw materials applied in this branch. In fact, the use of starch relative to cellulose and crude oil may increase since:

- the crude oil sources are dwindling;
- the price of cellulose fibres is more than double that of starch (Rexen et al. 1988);
- the cellulose supply is decreasing and will decrease further as long as pollution reduces the growth rate of the forests all over the world;
- starch can be produced until the market is saturated;
- the properties of granular starch can be changed via molecular engineering techniques, rendering starch more suitable for chemical and/or bio-technological processes. This requires insight in the factors influencing the applicability of starch (for instance the amount of protein, the ratio of amylose versus amylopectin and the structural features of the starch granules) and the development of techniques to manipulate these factors.

CHEMICAL COMPOSITION OF STARCH.

"Starch" is a multiple component system, mainly consisting of two different vegetable storage polysaccharides: amylose and amylopectin. Amylose is a linear chain of D-glucose units linked together by α-1,4 bonds (Figure 1). The length of amylose chains among different plant species is variable (Table I) but usually ranges between 10^2 to 10^4 glucose units (Shannon and Garwood 1984).

Amylopectin consists of short chains of, on the average, 20 to 30 α-1,4 linked D-glucose units which are cross-linked by α-1,6 bonds (see Figure 2). It may consist of a total of 10^4 to 10^5 glucose units (Shannon and Garwood 1984).

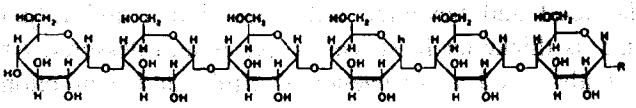

Figure 1: The structure of amylose.

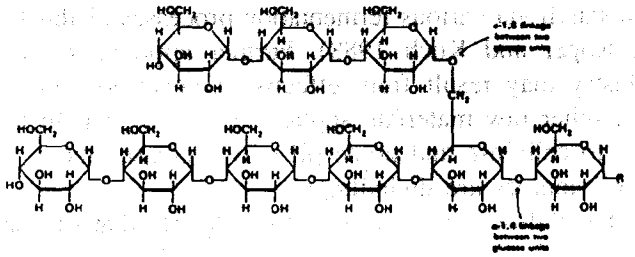

Figure 2: The structure of amylopectin.

Amylopectin is structurally similar to glycogen, the only storage polysaccharide found in bacteria (Fox et al. 1976), and mammals (Blumenfeld and Krisman 1986, Pitcher et al. 1987). Amylopectin contains fewer branch points than glycogen (Gunja-Smith et al. 1970). Moreover, the fine structure of glycogen is different from the fine structure of amylopectin. Glycogen consists of linear fragments of approximately the same length (Gunja-Smith et al. 1970), whereas amylopectin consists of three to five distinct classes of linear fragments (Gunja-Smith et al. 1970, Hizukuri 1986).

Amylose and amylopectin are deposited in starch granules together with small amounts of lipid, protein, and phosphorus (mainly present as phospholipids). The actual composition of starches varies between different plant species (in Table I data are given of the most important sources for industrial application). Physical parameters such as swelling power at 95°C, solubility in water at 95°C, pasting temperature, paste texture, clarity and viscosity, resistance to shear, and rate of retrogradation also differ for starches from different plant sources. It appears that these physical parameters are mainly determined by the lipid and amylose content of the starch granules, and the degree to which amylose is polymerized.

Lipids are complexed to amylose and their presence represses the swelling power and solubilization of starch granules at 95°C. Thus, fatty substances decrease the binding force and thickening power of starch pastes. Furthermore, pastes formed from corn starch are cloudy or opaque due to the presence of amylose-lipid complexes whereas pastes from potato starch are translucent. Finally, fatty acids give rise to undesirable tastes and flavors during the application of starch, due to oxidizing processes.

Additionally, starches may have a mealy flavor and odor and a tendency to foam building due to a high protein content.

Amylose molecules tend to retrograde (=precipitate) from aqueous solutions. This results for instance in the formation of skins on hot pastes. The rate of retrogradation depends on the amount of amylose, the degree of polymerization (the higher, the lower the rate of retrogradation) and the lipid content (the higher, the higher the rate of retrogradation).

Table I: Characteristics of commercially applied starches. Reprinted with permission of the author from the AVEBE brochure "Differences between commercial native starches" by J.J.M. Swinkels (1989). The table is continued on the next page.

Composition and Properties	Potato starch	Maize starch	Wheat starch	Tapioca starch	Waxy maize starch
Production (million tons)					
World	2	17	1.2	1.5	0.2
European Community (EC)	1.0	2.6	0.6	—	—
Main production countries	Holland, Sovjet-Union, Poland	USA, Japan, Sovjet-Union	EC, USA, Japan	Thailand, Brasil	USA

Starch Granule Properties					
Type of starch	tuber	cereal	cereal	root	cereal
Shape of granules	oval, spherical	round, polygonal	round, lenticular	truncated, round	round, polygonal
Diameter, range (μm)	5-100	2-30	0.5-45	4-35	2-30
Composition of starch granules (Average values)					
Moisture at 65% RH and 20 °C	19	13	13	13	13
Lipids (% on dry substance)	0.1	0.8	0.9	0.1	0.2
Nitrogen compounds (% on d.s.)	0.1	0.35	0.4	0.1	0.25
Ash (% on d.s.)	0.35	0.1	0.2	0.1	0.1
Phosphorus (% on d.s.)	0.08	0.02	0.06	0.01	0.01
Starch bound phosphorus	0.08	0.00	0.00	0.00	0.00
Pregelatinized starches					
Amount of taste and odor substances (relative)	low	high	high	very low	medium
Amylose and amylopectin					
Amylose content (% on d.s.)	21	28	28	17	1
Amylose, Degree of Polymerization (DP):					
Number average DP	4900	930	1300	2600	—
Weight average DP	6400	2400	—	6700	—
Apparent DP distribution	840-22,000	400-15,000	250-13,000	580-22,000	—
Amylopectin, Degree of Polymerization (DP) DP x 10^4 (Range)	0.3-3	0.3-3	0.3-3	0.3-3	0.3-3
Gelatinization characteristics					
Pasting temperature, °C	60-65	75-80	80-85	60-65	65-70
Swelling Power at 95 °C	1153	24	21	71	64
Solubility at 95 °C (%)	82	25	41	46	23
Properties of starch pastes					
Paste viscosity	very high	medium	low	high	high
Water-binding ability in parts of water per part of dry native starch to give the same hot viscosity after cooking	24	15	13	20	22
Paste texture	long	short	short	long	long
Past clarity	nearly clear	opaque	cloudy	quite clear	fairly clear
Resistance to shear	low	medium	medium	low	low
Rate of Retrogradation	medium	high	high	low	very low
Main commercial uses	food; paper; adhesives	sugars; paper; corrugated board	sugars; bakery-products	food; adhesives	food; adhesives

It is clear from Table I that potato starch has some very favourable characteristics: a low protein, lipid and amylose content together with a highly polymerized type of amylose. This results sometimes in the preference of potato starch to all other starches, since it has a high water binding capacity, forms clear pastes, retrogrades slowly, and has a bland flavor. In other applications *waxy* corn starch is preferred to potato starch since it is not subject to retrogradation at all, whereas potato starch retrogrades very slowly. However, if it would be possible to obtain potato starch without amylose, potato starch would be preferentially applied.

In the paper and adhesive industries starches with a high amylose content are preferred. Thus, it would be advantageous to increase the amount of amylose in potato starch to make it more suitable for the paper and adhesive industries.

To manipulate the production of amylose and amylopectin it is necessary to get insight in the enzymes involved in the biosynthesis of starch, and the ways in which these enzymes interact with one another *in vivo*. Such knowledge can be generated by characterizing starch mutants and by studying the biosynthetic pathways for amylose and amylopectin both *in vitro* and *in vivo*.

MUTATIONS AFFECTING STARCH COMPOSITION.

All normal reserve type starch granules consist roughly of 20-30 % amylose and 70-80 % amylopectin (Shannon and Garwood 1984), but many mutants with altered starch compositions have been described (Table II). The most drastic mutation is the complete loss of amylose as found in *waxy* maize (Echt and Schwartz 1981, Shure et al. 1983), *waxy* barley (Rohde et al. 1988), *waxy* sorghum, *waxy* amaranthus (Konishi et al. 1985), *glutinous* rice (Sano 1984, Villareal and Juliano 1986) and *amylose-free* potato (Hovenkamp-Hermelink et al. 1987, Chapter 2).

Maize mutants with an increased amylose content have also been described e.g. *dull*, *sugary*, and *amylose-extender* (Dickinson and Preiss 1969, Boyer and Preiss 1981, Hedman and Boyer 1982, Shannon and Garwood 1984). These mutations also affect the total amount of starch formed on a dry weight basis (Table II). *Shrunken* and *brittle* mutations are characterized by an accumulation of sugars at the expense of starch (Shannon and Garwood 1984). So far, all mutations affecting starch synthesis have been mapped on the plant genome (Eriksson 1969, Shannon and Garwood 1984, Yano et al. 1988).

Table II: Starch and amylose content of several maize mutants. Data are from Shannon and Garwood 1984.

genotype	% starch of dry weight	% amylose of starch*)	
wild type	76	29	27-29
waxy	69	0	0-1
dull	63	55	34-38
sugary	62	65	28-33
amylose-extender	49	33	57-61

*) the apparent amylose content as determined following gelfiltration chromatography (first row). When iodine binding procedures are used different values are obtained (second row).

Mutations affecting the protein production in maize endosperm are known as *opaque* (o2, o6 and o7) and *floury* (fl, fl2 and fl3) (Shannon and Garwood 1984). Mutants in lipid production have not been reported.

ENZYMES INVOLVED IN THE SYNTHESIS OF STARCH.

The formation of both amylose and amylopectin is mediated essentially by two enzymes: one enzyme that catalyzes the formation of α-1,4 bonds and another that synthesizes α-1,6 cross-links.

It was originally thought (Hanes 1940) that phosphorylase (EC 2.4.1.1) catalyzes the formation of α-1,4 bonds between the non-reducing ends of preexisting primers and glucose molecules:

$$\text{Glucose-1-P} + \text{glucose}_n \rightleftharpoons P_i + \text{glucose}_{n+1}$$

In 1961 Leloir and coworkers demonstrated that a UDP-Glc and/or ADP-Glc transglucosylase (EC 2.4.1.24) bound to the starch granule was also able to catalyze the elongation of preexisting primer molecules:

$$\text{ADP-glucose or UDP-glucose} + \text{glucose}_n \rightleftharpoons \text{ADP or UDP} + \text{glucose}_{n+1}$$

The substrate for this transglucosylase is formed by ADP-Glc or UDP-Glc pyrophosphorylases (EC 2.7.7.27) from Glc-1-P and ATP or UTP or via a reversal of the sucrose synthase reaction (de Fekete and Cardini 1964):

$$\text{sucrose} + \text{ADP or UDP} \rightleftharpoons \text{ADP-glucose or UDP-glucose} + \text{fructose}$$

Measurements of the *in vivo* concentrations of Glc-1-phosphate and P_i suggest that phosphorylase is not active in the synthesis of starch but in its breakdown (Preiss 1982). Thus, starch seems to be produced by a transglucosylase bound to the starch granule, rather than by phosphorylase action.

The granule-bound starch synthase(s).

The *in vitro* substrates of the granule-bound starch synthase are ADP-Glc and UDP-Glc, but ADP-Glc is the better donor based on K_m and V_{max} measurements (Vos-Scheperkeuter et al. 1986). The *in vivo* donor seems to be predominantly, or even exclusively, ADP-Glc since the amount of ADP-Glc pyrophosphorylase activity correlates well with the amount of starch synthesized in starch mutants from maize (Dickinson and Preiss 1969) and *Arabidopsis thaliana* (Lin et al. 1988).

Solubilization of the granule-bound starch synthase and subsequent ion-exchange chromatography revealed that maize starch granules contain two granule-bound starch synthases (MacDonald and Preiss 1985). It is not known if this is the case for all vegetable starches.

The granule-bound starch synthase is thought to be mainly responsible for the synthesis of amylose, since the protein is absent from *waxy* mutants (Echt and Schwartz 1981, Shure et al. 1983, Sano 1984, Konishi et al. 1985, MacDonald and Preiss 1985, Villareal and Juliano 1986, Vos-Scheperkeuter et al. 1986, Hovenkamp-Hermelink et al. 1987, Chapter 2). More direct evidence comes from experiments in which the gene coding for the granule-bound starch synthase is inhibited by antisense GBSS sequences (Visser et al. 1989). The amylose content of plants transformed with antisense sequences decreases as does the activity of the granule-bound starch synthase in starch granules isolated from such plants. Thus, granule-bound starch synthase activity is essential for the production of amylose.

Labeling experiments with isolated starch granules (Leloir 1961, Baba et al. 1987, Chapter 5) and tissue slices (Chapter 5) indicate that the granule-bound starch synthase elongates amylopectin as well as amylose chains. Furthermore, the solubilized granule-bound starch synthase was shown to use both amylose and amylopectin as primer (MacDonald and Preiss 1985). Thus the *in vivo* role of the granule-bound starch synthase seems to be more complex than expected from mutant analysis. Still, it can be concluded that the synthesis of amylose inside starch granules is a unique feature of the granule-bound starch synthase.

The soluble starch synthases.

Since the discovery of the ADP-Glc transglucosylase in starch granules (Leloir et al. 1961) a soluble transglucosylase using exclusively ADP-Glc as the substrate was identified in potato (Frydman 1963, Frydman and Cardini 1964, Frydman and Cardini 1966, Hawker et al. 1972), maize (Ozbun et al. 1971, Hawker and Downton 1974, Boyer and Preiss 1979, Pollock and Preiss 1980) and several other plants (Ozbun et al. 1971, Hawker and Downton 1974, Matters and Boyer 1981, Boyer and Fisher 1984, Boyer 1985).

Purification of this enzyme by ion-exchange chromatography revealed that the soluble transglucosylase generally consists of two forms (Hawker et al. 1972, Boyer and Preiss 1979, Pollock and Preiss 1980). One form (SSS-I from maize endosperm) has a relatively high affinity for primer molecules and prefers glycogen to amylopectin as a primer (MacDonald and Preiss 1985). High amounts (0.25-0.50 M) of sodium citrate or sodium malate stimulate the incorporation of glucose from ADP-Glc into amylopectin 5 fold, and induce the incorporation of Glc into trace amounts of endogenous primer molecules (Boyer and Preiss 1979). This form is called the citrate stimulated soluble starch synthase. The second form (SSS-II) binds more tightly to DEAE-cellulose, has a lower affinity for primer molecules, prefers amylopectin to glycogen and is stimulated to a much smaller extent by 0.5 M sodium citrate than the first form (MacDonald and Preiss 1985). It does not show activity in the absence of exogenously added primer molecules and is therefore called the "primed" soluble starch synthase. This soluble starch synthase is absent from maize leaves (Dang and Boyer 1988).

The granule-bound and soluble starch synthases are definitely different enzymes in maize, since immunological cross reactions are not observed and the molecular weights of both the enzymes differ (MacDonald and Preiss 1985). Comparable results were found for the potato starch synthases (Chapter 2 and 3).

The soluble starch synthases are thought to be predominantly involved in

the synthesis of the α-1,4 glucan linkages present in amylopectin, since amylopectin is the only product formed in *waxy* starch mutants. The different physiological roles of the two soluble starch synthases have never been investigated since mutations affecting one (or both) of the soluble starch synthases have never been reported.

The branching enzymes.

The α-1,6 cross links present in amylopectin are synthesized by branching (or Q-) enzyme activity. In maize endosperm three different branching isozymes were identified after ion-exchange chromatography (Boyer and Preiss 1981). They are called, in order of their elution, I, IIb and IIa. The branching enzymes IIa and IIb are immunologically related but differ from branching enzyme I (Fisher and Boyer 1983, Singh and Preiss 1985).

The only mutation known to result in loss of one of the branching enzymes in maize is the *amylose-extender*. This mutation interferes with the activity of branching enzyme IIb (Boyer and Preiss 1981, Hedman and Boyer 1982), resulting in a higher ratio of amylose to amylopectin (Table II), less highly branched amylopectin and an increased amount of sugars (Shannon and Garwood 1984). Wrinkled pea seeds also lack one isoform of branching enzyme activity, resulting in a comparable mutation (Smith 1988, Bhattacharyya et al. 1990).

Other plants have also been shown to contain multiple branching enzymes (Hawker et al. 1974, Matters and Boyer 1981, Boyer and Fisher 1984, Boyer 1985, Smyth 1988, Smith 1988, Goldner and Beevers 1989), but bacterial and mammalian cells seem to contain only one branching enzyme (Preiss 1982).

The physiological role and the necessity of multiple branching enzymes in maize is not understood. It has been suggested that SSS-I and branching enzyme IIb and SSS-II and branching enzyme IIa act as enzyme complexes (Preiss 1982), hence their comigration on native gels (Schiefer et al. 1973) and possibly also their coelution from DEAE-cellulose (Boyer and Preiss 1981). Amylopectin would then be the product of the simultaneous action of both enzymes in each complex and the asymmetric structure of amylopectin (Gunja-Smith 1970, Whelan 1971, Hizukuri 1986) might be due to the different products generated by the two enzyme complexes.

Thus far, only one branching enzyme has been identified in potato tubers (Drummond et al. 1972, Borovsky et al. 1975, Vos-Scheperkeuter et al. 1989). Based on immunological cross-reactions it can be said that the potato branching enzyme resembles the type I branching enzyme from maize (Vos-Scheperkeuter et al. 1989). It is not tightly complexed to soluble starch synthase since the enzymes can be separated by ion-exchange chromatography

(Chapter 3).

Regulation of starch synthesis.

Starch synthesis appears to be regulated at the level of substrate production. ADP-Glc pyrophoshorylase, the enzyme catalyzing the synthesis of substrate, is stimulated by 3-P-glyceraldehyde and inhibited by inorganic phosphate in several plant species (Preiss et al. 1985, Spilatro and Preiss 1987, Preiss et al. 1987). Thus, a high rate of photosynthesis (resulting in the production of 3-P-glyceraldehyde, and the utilization of inorganic phosphate via photophosphorylation) is tightly coupled to a high rate of starch synthesis. A low rate of photosynthesis is reversely coupled to a low rate of starch synthesis.

Additionally, inorganic pyrophosphate inhibits the activity of ADP-Glc pyrophosphorylase (Preiss et al. 1985, Preiss et al. 1987). It thereby possibly coordinates the biosynthetic pathways for cell growth and starch synthesis. During cell growth high amounts of pyrophosphate are produced as a byproduct of biosynthetic reactions. The pyrophosphate produced is not totally degraded by pyrophosphatase and inhibits starch synthesis to ensure the continuous supply of metabolites for cell growth (Preiss 1985). Lower levels of cell growth consequently coincide with lower levels of pyrophosphate and therefore with higher levels of starch synthesis.

Furthermore, experiments described by Oparka and Wright (1988, 1988a) suggest a regulatory influence of the turgor potential on starch synthesis.

Initiation of starch synthesis.

Granule-bound and soluble starch synthases need primer molecules for their activity. The smallest effective primer molecule is maltose (Leloir et al. 1961, MacDonald and Preiss 1985) and the question arises which molecules initiate the *in vivo* synthesis of starch and how these molecules are formed.

This question is common to the synthesis of a variety of polysaccharides such as glycogen, β-1,2-glucans, β-1,3-glucans, dextrans and glucosaminoglycans (Blumenfeld and Krisman 1986). Some reports indicate that polysaccharide synthesis is preceded by the formation of a glucoprotein (Lavintman and Cardini 1973, Lavintman et al. 1974, Krisman and Barengo 1975, Tandecarz et al. 1978, Sivak et al. 1981 and 1981a, Blumenfeld and Krisman 1986, Hanada and Takehara 1987, Pitcher et al. 1988, Andaluz et al. 1988).

Most of the mentioned reports deal with the initiation of glycogen synthesis, which is thought to be preceded by the formation of a 38 kDa glycoprotein (Pitcher et al. 1987, Lomako et al. 1988). A "glycogen initiator syn-

thase" catalyzes the attachment of a single glucose residue at position C1 to a unique tyrosyl residue in the glycoprotein (Smythe et al. 1988). The glycoprotein in turn autocatalyzes the incorporation of not more than 5 glucose residues, which are provided by UDP-Glc (Pitcher et al. 1988). Divalent cations are required for this reaction to occur (Pitcher et al. 1988). The glycoprotein formed, called glycogenin, then serves as the primer molecule of glycogen synthase.

Antibodies raised against rabbit muscle glycogenin react with proteins from muscle tissue from a wide variety of species as well as plant tissues including sweet corn, sweet potato, banana, cocoyams and cowpea seeds (Singh et al. 1988). This suggests that this protein might be a universal primer of glycogen and probably also of starch synthesis.

In proplastid fractions isolated from potato tuber a 38 kDa protein was identified that accepts, possibly by autocatalysis, a limited number of glucose units provided by UDP-Glc (Lavintman and Cardini 1973, Lavintman et al. 1974). It was furthermore shown that this 38 kDa glucoprotein primed a second transglucosylase in the proplastid fraction. This enzyme was stimulated by divalent cations (Lavintman et al. 1974). It is tempting to speculate on the relatedness between this 38 kDa protein, possibly priming starch synthesis, and the 38 kDa glycogenin priming glycogen synthesis.

PLASTIDS.

Different plastid types.

Starch is laid down in granules in specialized cellular organelles: chloroplasts and amyloplasts. Chloroplasts contain the green photosynthetic pigments, mainly chlorophylls, and carry on photosynthesis. A substantial part of the products of photosynthesis is transiently stored as starch. The starch formed during the day is degraded during the night or during periods when assimilation demand exceeds current photosynthetic production (Shannon and Garwood 1984). Alternatively, in many plants (principally monocots) carbohydrate is stored as sucrose, either in the photosynthetic cells or, as in sugarcane, in the vacuoles of special storage cells in the stem (Bidwell 1979).

Amyloplasts are found in the endosperm or perisperm of seeds and in vegetative organs like roots and tubers (Shannon and Garwood 1984). The organelles are specialized in the accumulation of reserve starch in storage cells. Amyloplasts also seem to play a role in the perception of gravity (Sack et al. 1983, Sack and Leopold 1985).

Both chloroplasts and amyloplasts develop from proplastids. Proplastids

can also differentiate into chromoplasts (containing pigments other than chlorophylls and not involved in photosynthesis), proteinoplasts (functional in protein deposition) and elaioplasts (organelles capable of accumulating oils and fats). The different types of plastids are to some extent interconvertible (Shannon and Garwood 1984, Zhu et al. 1984, Hansmann 1987, Keeling et al. 1988, Ngemprasirtsiri et al. 1988).

Translocators in the inner membrane of chloroplasts and amyloplasts.

All plastids are enclosed by two membranes, each membrane having the usual bilayer structure. Chloroplast membranes have been extensively studied to identify the molecules that are transported across the membranes.

The outer membrane is freely permeable to small molecules, but the inner membrane forms a barrier between the cytosol and the chloroplast stroma (Shannon and Garwood 1984). The inner membrane contains a phosphate translocator, facilitating the transport of dihydroxyacetone phosphate, glyceraldehyde-3-phosphate and inorganic phosphate (Fliege et al. 1978, Heber and Walker 1978, Preiss 1984, Thompson et al. 1987). The translocation of these compounds into the chloroplast is possibly coupled to the export of phosphorylated nucleotides (Heber and Santarius 1970). Hexose phosphates and pentose phosphates cross the inner membrane very slowly (Shannon and Garwood 1984). A dicarboxylate translocator, exchanging malate, oxaloacetate, succinate, α-ketoglutarate, fumarate and related amino acids between cytoplasm and stroma has also been identified (Shannon and Garwood 1984).

Since amyloplasts and chloroplasts are interconvertible (Zhu et al. 1984), it is assumed that the amyloplast membrane has a transport system that is similar to that found in chloroplasts. This implies that starch is formed from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate and that all the enzymes involved in the conversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate to Glc-1-phosphate are present in amyloplasts. In fact, this was demonstrated for amyloplasts isolated from soybean cells cultured in suspension (MacDonald and ap Rees 1983). Furthermore, labeling experiments showed that [U-^{14}C]glycerol was incorporated into starch indicating that there is a route from triose phosphate to starch in these cells (MacDonald and ap Rees 1983a).

However, the conversion of triosephosphates to starch is not a common feature of amyloplasts. Wheat amyloplasts, for instance, have been shown to lack plastidic fructose-1,6-biphosphatase (Entwistle and ap Rees 1988) and were shown to transport Glc-1-phosphate across the inner amyloplast membrane (Tyson and ap Rees 1988, Keeling et al. 1988). Transport measurements were also carried out with amyloplasts isolated from pea root

(Borchert et al. 1989). It appeared that the amyloplast membranes contain a phosphate translocator which transports inorganic phosphate, Glc-6-phosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate in a counter exchange mode. Glc-1-phosphate was not effectively transported (Borchert et al. 1989).

Transport of nuclearly encoded proteins into plastids.

Proplastids and chloroplasts are self-replicating (Bidwell 1979) and contain DNA and a protein-synthesizing capacity comparable to that of mitochondria (Ellis 1977). The genes thus far identified code either for components of the photosynthetic apparatus or for components for the transcription or translation of the plastome (Gounaris and Price 1987). Chromoplasts and amyloplasts share their DNA content with chloroplasts (Macherel 1985, Gounaris and Price 1987, Hansmann 1987), except in some cases for small modifications and/or methylated sequences (Hansmann 1987). However, the plastome seems not to be actively transcribed in plastids other than chloroplasts (Shannon and Garwood 1984, Gounaris and Price 1987, Hansmann 1987, Ngernprasirtsiri et al. 1988). Thus, most of the proteins present in chromoplasts and amyloplasts are nuclearly encoded (Shannon and Garwood 1984, Yano et al. 1988) and have to be transported into the plastids. Generally, such nuclearly encoded proteins contain a signal or transit peptide preceding the protein and determining its destination (Klösgen et al. 1986, Colman and Robinson 1986, Strzalka et al. 1987, Klösgen et al. 1989).

IN VIVO SYNTHESIS OF STARCH.

An understanding of the *in vivo* biosynthesis of starch and the assembly of starch granules requires an explanation for: (i) the existence of multiple isozymes for both the starch synthase and the branching enzyme; (ii) the relatively constant ratio of amylose to amylopectin; and (iii) the characteristic and constant morphology of the starch granule from different plant species (Fitt and Snyder 1984). Little is known about how plant genetic information is translated into a specific granule morphology (French 1984).

Various hypotheses have been developed to explain the simultaneous and apparently independent side-by-side synthesis of amylose and amylopectin. Erlander (1958) suggested that amylose and amylopectin are both formed from a common highly branched polymer (phytoglycogen). However *in vitro* experiments never gave rise to the production of normal amylopectin from glycogen (Marshall and Whelan 1970) and a "joining" enzyme, postulated to

be involved in the synthesis of amylose, was never shown to be present (Robyt 1984).

Alternatively, Whelan (1963) postulated that amylose and amylopectin are separated by a semi-permeable barrier. This barrier, which might even be amylopectin itself, would prevent the branching enzyme from acting on amylose but would not interfere with the diffusion of ADP-Glc to the site of amylose synthesis. Corroboration of this model comes from *in vitro* labeling experiments, showing that amylose is not extensively branched once it is laid down in the starch granule (Whistler and Young 1960).

Assuming that amylopectin results from the branching of amylose, Geddes and Greenwood (1969) proposed that intense synthesis of linear chains at, or near, the surface of the starch granule would completely saturate branching enzyme activity in that environment, permitting a portion of the linear molecules to remain unbranched. This hypothesis is based on the immobilization of the enzymes in relation to each other by adsorption to the starch granule surface, and the numerically higher amount of starch synthase molecules. Presently, no evidence is available on the relative amount of starch synthases and branching enzymes in any particular plant species. Thus verification of this hypothesis awaits further experiments.

French proposed that amylose and amylopectin molecules are both synthesized at the granule surface but in opposite directions: amylose molecules having their non-reducing ends, and amylopectin molecules having their reducing ends headed to the granule center (Robyt 1984). This model remains to be proven.

Finally, amylose may be the result of a specificity of branching enzyme activity for double helices. The amylose synthesized in the starch granules would never get the opportunity to form double helices since it is interspersed with amylopectin. Amylopectin on the other hand could easily form double helices and hence would be a good substrate for the branching enzyme (Borovsky 1975a, Robyt 1984, Mercier 1985).

More information about the *in vivo* synthesis of starch may result from the study of starch synthesis under (semi) *in vivo* conditions rather than from further analysis of the (partly) purified enzymes. In principle starch synthesis can be studied in isolated amyloplasts or in small tissue slices.

Isolation of amyloplasts.

Several procedures have been described for the isolation of amyloplasts. Some involve the production of protoplasts followed by gentle lysis (MacDonald and ap Rees 1983, Echeverria et al. 1985, Entwistle et al. 1988) but others consist only of a gentle homogenization step (Fishwick and Wright

1980, Liu and Shannon 1981a and 1981b, Gaynor and Galston 1983). In all cases amyloplasts have to be separated from other cellular components. This is most often achieved by differential centrifugation methods, in media with an extremely high specific density to support the amyloplasts. The procedures have been succesfully used in the isolation of maize (Echeverria et al. 1985), soybean (MacDonald and ap Rees 1983), and wheat amyloplasts (Entwistle et al. 1988). Amyloplasts were isolated from relatively young tissues and 25 to 50 % of the organelles were intact as measured by latency experiments.

So far, the isolation procedures for amyloplasts from mature potato tubers were less satisfactory and have not yielded more than 16 % intact organelles on a starch weight basis (Fishwick and Wright 1980). This percentage was increased to 27 % by the use of very young potato tubers (Mohabir and John 1988).

Employment of tissue slices in the elucidation of biosynthetic routes.

Permeabilized tissues have been succesfully used in elucidating the biosynthetic routes of cell walls in fungi (Farkas 1979). Tissue slices have also been used in the study of starch synthesis in leaf disks (Herold 1978), β-glucan synthesis in pea epicotyls (Raymond et al. 1978), glucan biosynthesis in red beet roots (Wasserman et al. 1985), triacylglycerol biosynthesis in sunflower cotyledons (Griffiths et al. 1988) and sucrose metabolism in potato tuber (Mohabir and John 1988, Oparka and Wright 1988, Wright and Oparka 1989). Here we will report on the use of tissue slices in the elucidation of starch synthesis in potato tubers.

Starch granule formation.

Another problem in understanding the *in vivo* synthesis of starch is the formation of the starch granule. Radiochemical experiments indicate that starch granules grow by apposition (Badenhuizen and Dutton 1956) but little information is available as to the initiation of starch granule formation. Badenhuizen reported that the first step may be the formation of a separate phase of amorphous starch that crystallizes at a certain moment and becomes the center of the developing starch granule (French 1984). This center may consist of amylose complexed to lipid, escaping from branching enzyme activity, which is sometimes visible as a blue "core" in *waxy* starch granules (French 1984). These lipid-amylose complexes may result from the specific binding and precipitation of amylose by lipoproteins, comparable to fungal starch synthesis (McCracken 1974, McCracken and Rutherford 1980, Varkey et al. 1985, Rutherford et al. 1986).

SCOPE OF THIS THESIS.

The research project described in this thesis was set up to obtain information about the *in vivo* synthesis of starch in potato tubers. To do so it was necessary to know which enzymes mediate the synthesis of starch in potato tubers. It appeared that potato tubers contain at least one granule-bound starch synthase (Vos-Scheperkeuter et al. 1986, Chapter 2), possibly two soluble starch synthases (Chapter 3) and only one branching enzyme (Vos-Scheperkeuter et al. 1989).

To study the *in vivo* role of these enzymes it was necessary to define a semi *in vivo* system, accessible to experimental manipulation and to define criteria to measure the enzymes involved in starch biosynthesis specifically. In Chapter 4 criteria are described which permit the specific measurement of the enzymes involved in the biosynthesis of starch. Furthermore, a semi *in vivo* system is described, based on the permeabilization of potato tuber tissue slices, which permits the specific measurement of starch synthases in the absence of branching enzyme activity.

In Chapter 5 these specific assay conditions are employed to define the primer molecules of the different starch synthase activities under semi *in vivo* conditions, without interference of the branching enzyme. The results indicate that granule-bound as well as soluble starch synthases elongate amylose and amylopectin. Both the starch synthases seem to prefer amylose as the substrate. Permitting branching enyzme activity by lowering the pH of the assay mixture gave rise to a higher specific labelling of the amylopectin molecules. Thus, amylose synthesis precedes amylopectin synthesis.

The data obtained are summarized in a model describing and hypothesizing about the side-by-side synthesis of amylose and amylopectin (Chapter 5 and 6).